Developmental Biology 381 (2013) 276–285

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Evolution of Developmental Control Mechanisms

The *ascl1a* and *dlx* genes have a regulatory role in the development of GABAergic interneurons in the zebrafish diencephalon

Ryan B. MacDonald [a,1,2], Jacob N. Pollack [a,1], Mélanie Debiais-Thibaud [a,3], Eglantine Heude [a], Jared Coffin Talbot [b,4], Marc Ekker [a,*]

[a] Center for Advanced Research in Environmental Genomics, Department of Biology, University of Ottawa, Ottawa, ON, Canada, K1N 6N5
[b] Institute of Neuroscience, 1254 University of Oregon, Eugene, OR, USA

ARTICLE INFO

Article history:
Received 18 February 2013
Received in revised form
8 May 2013
Accepted 25 May 2013
Available online 4 June 2013

Keywords:
ascl1a
dlx
gad1b
GABAergic interneuron
Telencephalon
Diencephalon

ABSTRACT

During development of the mouse forebrain interneurons, the *Dlx* genes play a key role in a gene regulatory network (GRN) that leads to the GABAergic phenotype. Here, we have examined the regulatory relationships between the *ascl1a*, *dlx*, and *gad1b* genes in the zebrafish forebrain. Expression of *ascl1a* overlaps with *dlx1a* in the telencephalon and diencephalon during early forebrain development. The loss of Ascl1a function results in a loss of *dlx* expression, and subsequent losses of *dlx5a* and *gad1b* expression in the diencephalic prethalamus and hypothalamus. Loss of Dlx1a and Dlx2a function, and, to a lesser extent, of Dlx5a and Dlx6a, impairs *gad1b* expression in the prethalamus and hypothalamus. We conclude that *dlx1a/2a* act downstream of *ascl1a* but upstream of *dlx5a/dlx6a* and *gad1b* to activate GABAergic specification. This pathway is conserved in the diencephalon, but has diverged between mammals and teleosts in the telencephalon.

Introduction

Gene regulatory networks (GRNs) are made up of dynamic interactions between transcription factors and *cis*-regulatory elements (CREs) found within the genome (for reviews see: Levine and Davidson (2005) and Davidson and Levine (2008)). CREs are classically thought to be non-coding regulatory sequences, comprised of clustered transcription factor binding sites; the binding of these transcription factors are able to affect the transcription of specific genes (for reviews see: Kulkarni and Arnosti (2003), Kadonaga (2004) and Panne (2008)). The overall levels and timing of gene expression are conferred by the cumulative contributions of multiple transcription factors on a myriad of regulatory regions. The genes regulated by this process during development often encode transcription factors that will play a role in the regulation of other transcription factor genes located downstream in the GRN, eventually resulting in the expression of terminal differentiation genes leading to a specified cell type.

Ascl1 (*Mash1*) is one of the basic helix–loop–helix (bHLH) transcription factors thought to play important roles in GRNs controlling neurogenesis (for reviews see: Bertrand et al. (2002) and Allan and Thor (2003)). *Ascl1* is expressed in proliferating neural precursors in the subpallial telencephalon and prethalamus of the mouse (Lo et al., 1991; Guillemot and Joyner, 1993; Porteus et al., 1994; Yun et al., 2002; Andrews et al., 2003). *Ascl1* mutants have defects in neural specification and in the timing of differentiation in the ventral forebrain, including altered telencephalic expression of the *Dlx* genes and *Gad1* (*Gad67*), which encodes glutamic acid decarboxylase, the enzyme responsible for the production of γ-amino butyric acid (GABA) (Casarosa et al., 1999; Horton et al., 1999: Yun et al., 2002; Long et al., 2009a). Ectopic expression of *Ascl1* leads to *Gad1* expression in the mouse dorsal telencephalon, further supporting a role for *Ascl1* in GABAergic interneuron development (Fode et al., 2000). In zebrafish there are two *Ascl1* orthologs, *ascl1a* and *ascl1b*. These genes are expressed in the embryonic forebrain, including the subpallial telencephalon and prethalamus, reminiscent of *Ascl1* expression in the mouse (Allende and Weinberg, 1994; Wullimann and Mueller, 2002).

In the mouse forebrain, expression of *Ascl1* and *Dlx* genes overlap suggesting these genes may genetically interact during mouse forebrain development (Porteus et al., 1994; Yun et al., 2002; Andrews et al., 2003). Consistent with this hypothesis,

* Corresponding author. Fax: +1 613 562 5486.
E-mail address: mekker@uottawa.ca (M. Ekker).
[1] Denotes equal contribution.
[2] Present address: Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3DY, UK.
[3] Present address: Institut des Sciences de l'Evolution, Université de Montpellier II, UMR5554, Montpellier Cedex, France.
[4] Present address: Department of Molecular and Cellular Biochemistry and Department of Molecular Genetics, Ohio State University, 1060 Carmack Rd, Columbus, OH 43219, USA.

0012-1606/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2013.05.025

Ascl1$^{-/-}$ mutant mice have mis-expression of *Dlx* in the ganglionic eminences (Casarosa et al., 1999; Horton et al., 1999; Yun et al., 2002; Long et al., 2009a) and ASCL1 proteins have been shown to activate and directly bind to a *Dlx1*/*Dlx2* regulatory element (Poitras et al., 2007).

The *Dlx* genes encode homeodomain transcription factors expressed in the ganglionic eminences of the telencephalon and diencephalon in the mouse. More specifically, four *Dlx* genes are expressed in the forebrain of the mouse: *Dlx1*, *Dlx2*, *Dlx5*, and *Dlx6* (Liu et al., 1997; Yang et al., 1998; Anderson et al., 1997a; Eisenstat et al., 1999), while five orthologous *dlx* genes are expressed in the forebrain of the zebrafish: *dlx1a*, *dlx2a*, *dlx5a*, *dlx6a*, and *dlx2b* (Akimenko et al., 1994; Ellies et al., 1997; Hauptmann and Gerster, 2000). The *Dlx* genes are expressed in highly overlapping but also distinct domains within the forebrain of mice and zebrafish, often correlating with neuronal differentiation and *Gad* expression (Liu et al., 1997; Eisenstat et al., 1999; MacDonald et al., 2010a; Stühmer et al., 2002a,b; Yun et al., 2002). Functional studies have shown that the *Dlx* genes are required for the differentiation and migration of most GABAergic neurons in the telencephalon and diencephalon (Anderson et al., 1997a,b; Stühmer et al., 2002a,b; Long et al., 2007; Long et al., 2009a,b; Wang et al., 2012). Additionally, DLX1 and DLX2 are involved in the suppression of neurite growth and branching, thus enabling the proper tangential migration of GABAergic neurons (Cobos et al., 2007).

The zebrafish *dlx* genes are involved in branchial arch and sensory placode development (Solomon and Fritz, 2002; Kaji and Artinger, 2004; Walker et al., 2006; Jackman and Stock, 2006; Sperber et al., 2008; Talbot et al., 2010), as are the mouse *Dlx* genes (Qiu et al., 1995; Depew et al., 2002; Jeong et al., 2008). However, despite their common use as forebrain markers, there has been little functional analysis of the *dlx* genes in the zebrafish brain. To characterize the role of *ascl1a* and *dlx* in the GRN(s) controlling GABAergic interneuron differentiation in the zebrafish forebrain, we have knocked down their function and assayed the effects on downstream targets. Our results show that the *ascl1a* gene regulates *dlx* genes necessary for proper *gad1b* expression in the diencephalon of the zebrafish. Thus, these genes are key part of a GRN involved in early forebrain development that is conserved among bony vertebrates.

Materials and methods

Zebrafish strains and staging

Embryos were obtained and housed using standard procedures described in Westerfield (2000). The following transgenic zebrafish lines were used in this study: *Tg*(*dlx1a*/*2aIG:GFP*) (MacDonald et al., 2010a), *Tg*(*dlx1URE2:GFP*) (MacDonald et al., 2010b), and *Tg*(*dlx5a*/*6a:GFP*) (Ghanem et al., 2003). All developmental stages are reported as hours post-fertilization (hpf) All experiments were performed in accordance with the Canadian Council on Animal Care guidelines and approved by institutional animal care committees.

Morpholino and mRNA injections

Morpholino oligonucleotides (MO) were injected into one-cell stage wild type or transgenic zebrafish embryos at concentrations ranging from 2 to 4 ng/μl. The following translation blocking morpholinos were used: *dlx1a* (Sperber et al., 2008), *dlx2a* (Sperber et al., 2008), *dlx2b* (Jackman and Stock, 2006), *dlx5a* (Walker et al., 2006), *dlx6a* (5′TGGTCATCAT–CAAATTTTCTGCTTT3′). The *ascl1a*$^{5'UTR}$ MO (Cau and Wilson, 2003) was kindly provided by Dr. S. Wilson. Splice blocking MOs for *dlx5a* (Talbot et al., 2010) were kindly provided by Dr. C. B. Kimmel, and were used to confirm the translation blocking MO phenotypes. The *dlx6a* splice-blocking morpholino binds to the end of the second exon and inhibits the splicing of the second intron (5′AAATGAGTTCACATCTCACCTGCGT3′).

In situ hybridization and imaging

Whole mount mRNA *in situ* hybridization was carried out as described in Thisse and Thisse (1998). The antisense mRNA probes were labeled with digoxygenin-11-UTP (Roche, 11277073910) and synthesized from the following cDNA clones: *dlx1a* (Ellies et al., 1997), *dlx2a* (Akimenko et al., 1994), *dlx5a* (Akimenko et al., 1994), *dlx6a* (Ellies et al., 1997), *dlx2b* (Ellies et al., 1997), *gad2* (Martin et al., 1998), *gad1b* (Mueller et al., 2008), *ascl1a* (Cau et al., 2000), *nkx2.1a* (Rohr and Concha, 2000), *emx2* (Morita et al., 1995), *lhx5a* (Toyama et al., 1995), *gfp* (Dorsky et al., 2002). After the procedure, embryos were post fixed in 4% PFA and cleared overnight in glycerol.

Fluorescent RNA *in situ* hybridization was carried out with a protocol modified from those described previously (Jowett and Yan, 1996; Welten et al., 2006; Talbot et al., 2010). The DNP-labeled probe was revealed with tyr-Cy5, whereas dig-labeled probes were revealed using tyr-Cy3. Fluorescein-labeled probes were revealed with tyr-fluorescein (available from Perkin-Elmer). The full tissue labeling protocols can be found online: http://wiki.zfin.org/display/prot/Triple+Fluorescent+In+Situ.

For confocal imaging, embryos were placed in mounting media on glass slides and positioned under coverslips. Confocal z-stacks were obtained by using a Zeiss LSM5 PASCAL (Carl Zeiss, Germany) with an excitation laser at 488 (Fluorescein), 543 nm (Cy3), and 633 nm (Cy5).

Rescue experiments and morphant phenotype scoring

For exogenous expression of *dlx* genes, capped full-length mRNA was synthesized *in vitro* using linearized PCS2+ plasmids (mMessageMachine; Ambion) and purified. The following plasmids as templates: mut*dlx2a* (mutagenized at MO binding site) and mut*dlx5a* (Supplementary Table 1). A solution containing 40 ng/μl of mRNA, along with MO, was co-injected into single cell embryos. Individuals were classified and scored in two groups: either as having reduced or normal prethalamic expression. Embryos from each treatment were scored in a double-blind manner and plotted with standard error from three individual experiments. One way ANOVA was used to compare data.

Results

The ascl1a, dlx, and gad1b genes are co-expressed in the forebrain

We utilized triple fluorescent *in situ* hybridizations to determine if the zebrafish *ascl1a*, *dlx* and *gad* genes show overlapping expression in the forebrain as they do in the mouse. The expression of *ascl1a* begins in the prospective forebrain at 10 hpf and lasts until at least 72 hpf (Allende and Weinberg, 1994). The *dlx1a* and *dlx2a* (hereafter called *dlx1a*/*2a*) genes are expressed starting at 13 hpf in the prospective forebrain (Akimenko et al., 1994; Ellies et al., 1997). At 24 hpf, *dlx1a* is expressed in the telencephalon and two domains of the diencephalon, the prethalamus (or ventral thalamus) and the hypothalamus (Fig. 1A). The *dlx2a* expression domains are identical to *dlx1a* (MacDonald et al., 2010a), so we consider *dlx1a* expression as representative of the two genes. At 24 hpf, expression of *ascl1a* is detected in the telencephalon and prethalamus, and partially overlaps with the *dlx1a* expression

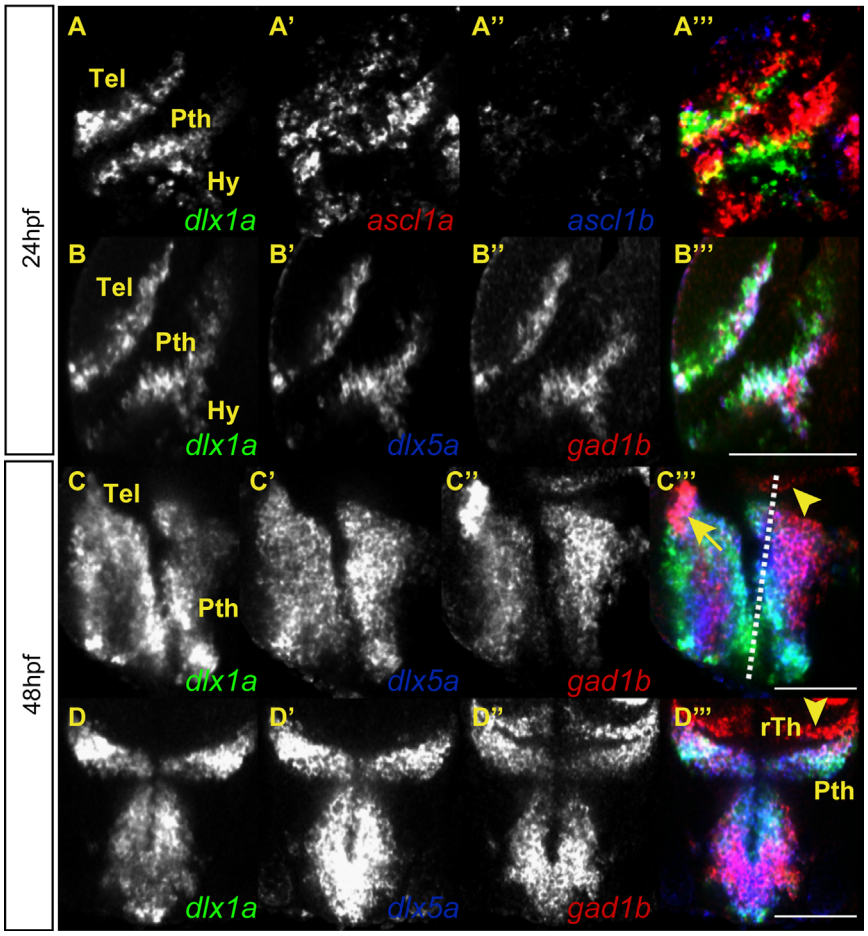

Fig. 1. The expression domains of the *ascl1a*, *dlx*, and *gad1b* genes overlap in the forebrain of the embryonic zebrafish. Single z sections on triple fluorescent *in situ* hybridizations. (A) In the forebrain lateral view of a WT embryo, the *dlx1a* and *ascl1a* are co-expressed throughout the telencephalon and prethalamus at 24 hpf, while *ascl1b* is not co-expressed at this stage. (B) The *dlx1a*, *dlx5a*, and *gad1b* genes are co-expressed in the telencephalon, prethalamus, and hypothalamus at 24 hpf. (C) The *dlx1a*, *dlx5a*, and *gad1b* genes continue to be co-expressed in the telencephalon and prethalamus at 48 hpf. The *gad1b* expression is increased in the dorsal tip of the telencephalon (arrow). The plane of section for (D) is shown as a dotted line in C‴. (D) Cross-section showing the co-expression of *dlx1a*, *dlx5a*, and *gad1b* in the prethalamus (arrow). The rostral thalamus (arrowhead) is positive for *gad1b* expression but not *dlx1* or *dlx5a*. Tel = telencephalon, Pth = prethalamus, Hy = hypothalamus, rTh = rostral thalamus. Scale bar in A–D = 100 μm and in E = 40 μm.

domain (Fig. 1A). The *ascl1b* gene is paralogous to *ascl1a*, but each has unique and overlapping expression domains with the central nervous system, including the forebrain (Allende and Weinberg, 1994). At 24 hpf, expression of *ascl1b* was detected in the forebrain but had very little co-expression with *dlx1a* or *ascl1a* positive cells (Fig. 1A), indicating that *ascl1b* cannot activate *dlx* gene expression at this stage in the forebrain.

The *Dlx* genes are expressed in very similar domains within the developing forebrain of mice and zebrafish (Liu et al., 1997; Anderson et al., 1997a; Eisenstat et al., 1999; Akimenko et al., 1994; Ellies et al., 1997; Zerucha et al., 2000; Mueller et al., 2008; MacDonald et al., 2010a). At 24 hpf, the expression of *dlx5a* and *gad1b* are both highly overlapping with *dlx1a* expression in the telencephalon, prethalamus, and hypothalamus (Fig. 1B; MacDonald et al., 2010a). Expression of *dlx1a*, *dlx5a*, and *gad1b* remains highly overlapping at 48 hpf in the telencephalon (Fig. 1C), and prethalamus (Fig. 1C and D, arrows). However, there is an area of intense staining for *gad1b* in the dorsal region of the telencephalon potentially corresponding to GABAergic interneurons that will migrate into the pallium starting at approximately 72 hpf (Fig. 1C‴, asterisk) (Mione et al., 2008). Additionally, there is an area dorsal to the *dlx1a* and *dlx5a* expression domains of the prethalamus that is *gad1b* positive and *dlx* negative (Fig. 1D, arrowhead) that may correspond to the rostral thalamus (Peukert et al., 2011; Lauter et al., 2013).

Knockdown of ascl1a reveals a role in the regulation of the Dlx and gad1b genes in the prethalamus and hypothalamus

To determine the role of *ascl1a* in regulating *dlx* and *gad1b* gene expression in the zebrafish forebrain, we knocked down Ascl1a activity with a translation blocking morpholino (Cau and Wilson, 2003). Morpholino knock down of Ascl1a function in the zebrafish affects the development of the pituitary, neurogenesis in the epiphysis, and regeneration in the retina (Cau and Wilson, 2003; Herzog et al., 2004; Pogoda et al., 2006; Fausett et al., 2008), here we examine the consequences of its knockdown on GABAergic fate specification in the forebrain. Injected embryos were examined at 24 and 48 hpf, stages when the genes are co-expressed and the distinct regions of the forebrain are evident. In *ascl1a* morphants, there is a loss of *dlx1a*, *dlx2a* and *dlx5a* expression in the domain of the prethalamus and hypothalamus at 24 and 48 hpf (Fig. 2A–C, Supplementary Fig. 1, asterisks). A slight reduction in *dlx1a*, *dlx2a* and *dlx5a* expression in the telencephalon is also possible but difficult to assess by *in situ* hybridization. Expression of *gad1b* is also impaired in the ventral prethalamus and hypothalamus of *ascl1a* morphants at 24 and 48 hpf (about 65% of injected embryos; Fig. 2D, Fig. 3A, B and Supplementary Fig. 2A, B, G, H), suggesting the loss of either *ascl1a* or *dlx* gene function may play a role in the differentiation of *gad1b* expressing cells.

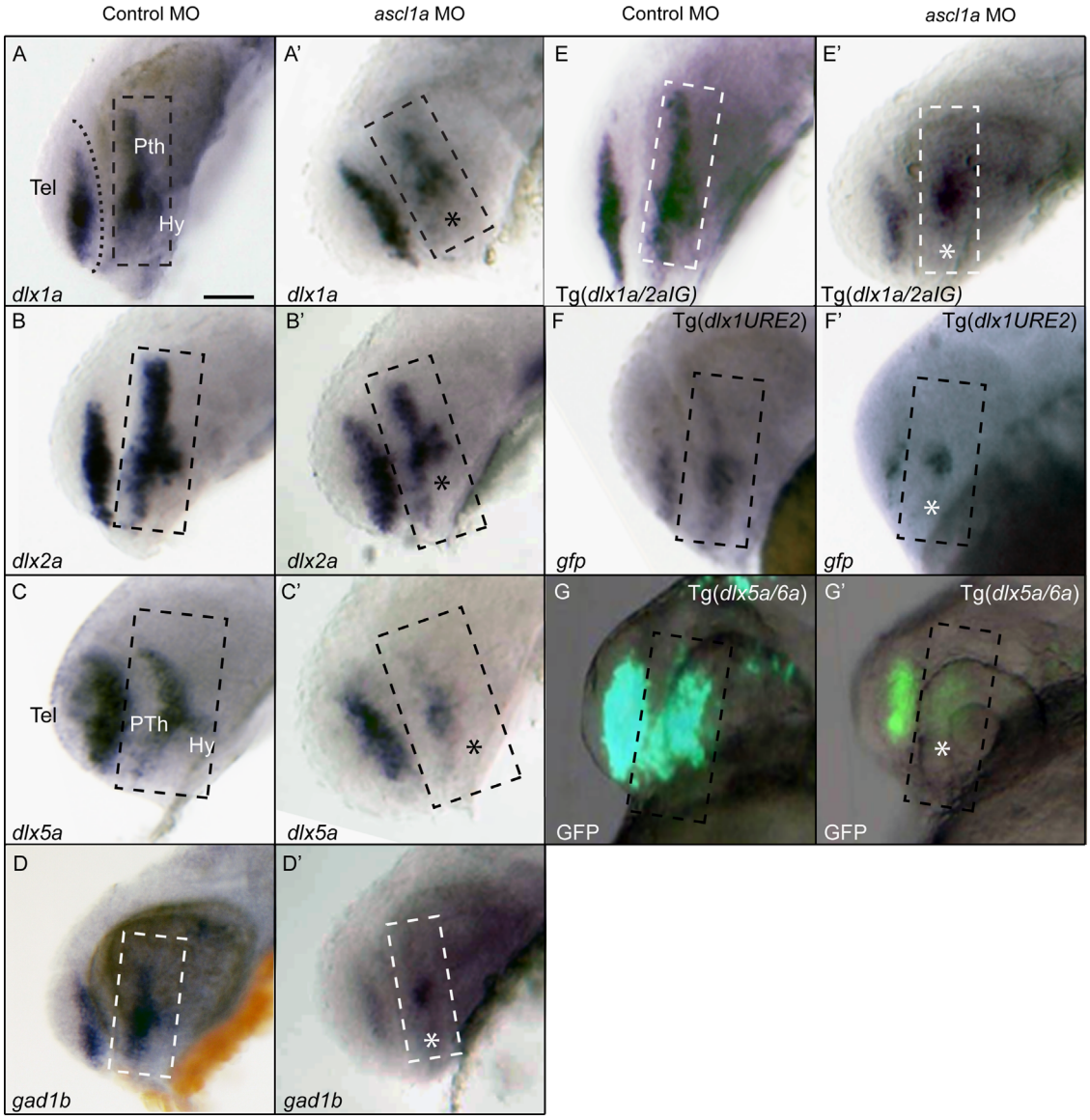

Fig. 2. Ascl1a function is required for proper expression of the *dlx* and *gad1b* genes in the forebrain at 24 hpf. (A, B) Expression of *dlx1a* and *dlx2a* is reduced in the prethalamus and hypothalamus (asterisk), but not affected in the telencephalon of *ascl1a* morphants compared to control embryos. (C) The expression of *dlx5a* is particularly reduced in the prethalamus and hypothalamus in *ascl1a* morphants. (D) *gad1b* expression is unaffected in the telencephalon, but reduced in the prethalamus and hypothalamus. (E) *Tg*(*dlx1a*/*2aIG*:*GFP*) embryos reduced *gfp* mRNA expression in the prethalamus of ascl1a morphants but telencephalic expression appears unaffected. (F) *Tg* (*dlx1URE2*:*GFP*) embryos show reduced *gfp* mRNA expression in the telencephalon and prethalamus. (G) The loss of GFP expression in the *Tg*(*dlx5a*/*6a*:*GFP*) transgenic line is consistent with a loss of *dlx5a* expression. Dashed box represents prethalamus region. Tel, telencephalon; Pth, prethalamus; Hy, hypothalamus. Scale bar = 50 μm.

Expression of *Dlx1*/*Dlx2* genes in the forebrain is conferred by at least two conserved CREs, I12b and URE2 whereas that of *Dlx5*/*Dlx6* is conferred at least by CREs located in the intergenic region (Zerucha et al., 2000; Ghanem et al., 2003; Ghanem et al., 2007; Potter et al., 2009; MacDonald et al., 2010a,b). To test if the loss of *ascl1a* function affects *dlx* gene regulation by altering the activity of *dlx* regulatory elements, we knocked down Ascl1a function in the following transgenic lines: *Tg*(*dlx1a*/*2aIG*:*GFP*) (MacDonald et al., 2010a), *Tg*(*dlx1aURE2*:*GFP*) (MacDonald et al., 2010b), and *Tg*(*dlx5a*/*6a*:*GFP*) (Ghanem et al., 2003). Both the *Tg*(*dlx1a*/*2aIG*:*GFP*) (Fig. 2E) and *Tg*(*dlx1URE2*:*GFP*) (Fig. 2F) embryos injected with the *ascl1a* MO show a reduced reporter gene expression in the prethalamus consistent with the loss of *dlx1a*/*2a* expression in this domain. The expression of *gfp* in *Tg*(*dlx1URE2:GFP*) is also reduced in the telencephalon indicating *ascl1a* is also necessary for proper regulation of *dlx1a* and/or *dlx2a* in this part of the forebrain (Fig. 2F). The *Tg*(*dlx5a*/*6a:GFP*) *ascl1a* morphants showed a severe loss of GFP expression in the prethalamus (Fig. 2G), while expression in the telencephalon may be reduced but is still detectable. Overall, our data support the hypothesis that *ascl1a* acts as an upstream regulator of the *dlx1a*, *dlx2a*, *dlx5a* and *gad1b* genes in the embryonic prethalamus and hypothalamus.

Knockdown of dlx paralogs results in the loss of gad1b expression in the forebrain

In the mouse, *Gad1* is downstream of *Dlx1* and *Dlx2* (Stühmer et al., 2002a; Long et al., 2007; Long et al., 2009a), prompting us to test this relationship in zebrafish. To assay a possible role for *dlx* genes in *gad1b* expression (orthologous to mouse *Gad1*) in the zebrafish forebrain at 24 and 48 hpf, we used translation and splice blocking MOs against *dlx1a*, *dlx2a*, *dlx5a*, and *dlx6a*. Single *dlx* morphants show no discernible changes in *gad1b* expression in the telencephalon, prethalamus or hypothalamus at 24 and 48 hpf (Supplementary Figs. 2D and 3A–C, E, F). At 48 hpf, *gad1b* expression in the telencephalon of *dlx1a*/*2a* double morphants is similar

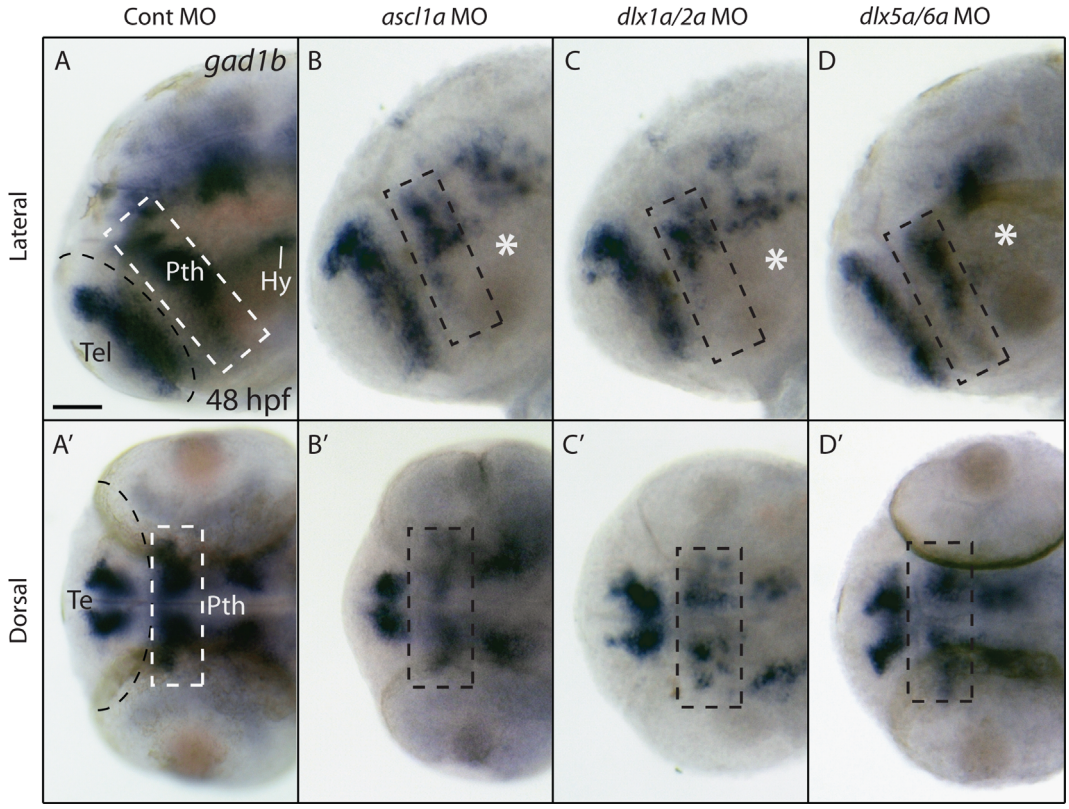

Fig. 3. The expression of *gad1b* is impaired in the prethalamus but not in the telencephalon of *ascl1a* morphants and of *dlx1a*/*2a* double morphants at 48 hpf. Lateral (A–D) and dorsal views (A′–D′) of the forebrain of control and morphant embryos. (A) Expression of *gad1b* in embryos injected with the control morpholino is seen in the telencephalon (Tel), prethalamic (Pth) and hypothalamic (Hy) diencephalon. (B) Morpholino knockdown of *ascl1a* results in a loss of ventral prethalamic (dashed box) and hypothalamic (asterisk) *gad1b* expression. (C) Double morpholino knockdown of *dlx1a*/*2a* results in decreased prethalamic and hypothalamic *gad1b* expression. (D) In *dlx5a*/*dlx6a* morphants there is mild reduction of prethalamic and a decrease in hypothalamic *gad1b* expression similar to that observed in *ascl1a* and *dlx1a*/*2a* morphants. Dashed line indicates telencephalon-diencephalon boundary. Scale bar = 50 μm.

to controls, but there is a reduction in the ventral prethalamus and in the hypothalamus (Fig. 3C and Supplementary Fig. 2C). The combinatorial loss of *dlx5a*/*6a* results in a mild reduction in prethalamic *gadb1* signal compared to *dlx1a*/*2a* morphants, and a reduction in the hypothalamic *gad1b* expression that is comparable to that observed in *ascl1a* and *dlx1a*/*2a* morphants. (Fig. 3D and Supplementary Fig. 2E). Finally, triple knockdown of the paralogs *dlx1a*, *dlx2a* and *dlx2b* does not increase the severity of *gad1b* loss in the prethalamus or result in any noticeable loss in the telencephalon, prethalamus or hypothalamus (Supplementary Fig. 3D). Therefore, *dlx1a*/*2a* and, possibly, *dlx5a*/*6a* are necessary for proper expression of *gad1b* in the prethalamus and hypothalamus. In *Dlx1*/*2*$^{-/-}$ mutant mice the expression of *Ascl1* was altered in regions of the telencephalon (Yun et al., 2002; Long et al., 2009a); we examined *ascl1a* expression in *dlx1a*/*2a* and *dlx5a*/*6a* double morphants. We did not observe any noticeable changes in *ascl1a* expression in the forebrain in these morphants (Supplementary Fig. 4).

The mouse *Dlx* genes are involved in auto- and cross-regulatory interactions in the telencephalon (Zerucha et al., 2000; Zhou et al., 2004; Poitras et al., 2007; Bond et al., 2009; Potter et al., 2009) and diencephalon (Long et al., 2009a). To test if the zebrafish *dlx1a*/*2a* genes play a role in the regulation of the *dlx5a*/*6a* bigene cluster, we examined *dlx5a* expression and the activity of *Tg*(*dlx5a*/*6a*:*GFP*) following MO-mediated knock down of *dlx1a* and/or *dlx2a*. There is no loss of *dlx5a* expression in single *dlx1a* or *dlx2a* morphant embryos (data not shown). In embryos injected with both MOs, there is a severe reduction of both telencephalic, prethalamus and hypothalamic expression of *dlx5a*, consistent with the loss of GFP expression in *Tg*(*dlx5a*/*6a:GFP*)(Fig. 4A–B). To confirm the observed differences in expression are not due to alteration of the anatomy of the telencephalon or diencephalon, we verified the expression of marker genes such as *nkx2.1a* (Fig. 4C), *lhx5a* (Fig. 4D), and *emx2* (Fig. 4E) and their expression domains are not markedly changed after the knockdown of *dlx1a*/*2a*. The *gad2* gene is also expressed in the telencephalon and prethalamus, in a pattern very similar to *gad1b* (Martin et al., 1998). However, *dlx1a*/*2a* morphants show no change in the expression of *gad2* at 24 hpf, indicating the *gad1b* and *gad2* genes may be regulated differently (Fig. 4F). To verify that changes in *gad1b* expression pattern in morphants is not due to cell death in the forebrain we stained double morphants with acridine orange and did not observe any overt increase in cell death (Supplementary Fig. 5).

Exogenous administration of dlx2a and dlx5a mRNA in ascl1a morphants partially rescues diencephalic gad1b expression

Rescue experiments were carried out to determine whether *ascl1a* and *dlx1a*/*2a* act sequentially or in parallel cascades to regulate prethalamic *gad1b* expression. We used exogenous expression of *dlx2a* or *dlx5a* mRNA to rescue the *gad1b* phenotype in the forebrain of *ascl1a* morphants. Rescue experiments were carried out by co-injecting *ascl1a* MO with *dlx2a* or *dlx5a* mRNA or co-injecting the *dlx1a*/*2a* MOs with mismatched *dlx2a* or *dlx5a* mRNA, and performing *in situ* hybridization for *gad1b* at 48 hpf. As both genes in a *dlx* bigene pair have highly redundant expression patterns and, possibly, biochemical function, we injected mRNA from only one gene of each pair. The *gad1b* phenotype of a given individual was classified as being “normal” (resembling the wild type expression, blue bars in Fig. 5), or “reduced” in the

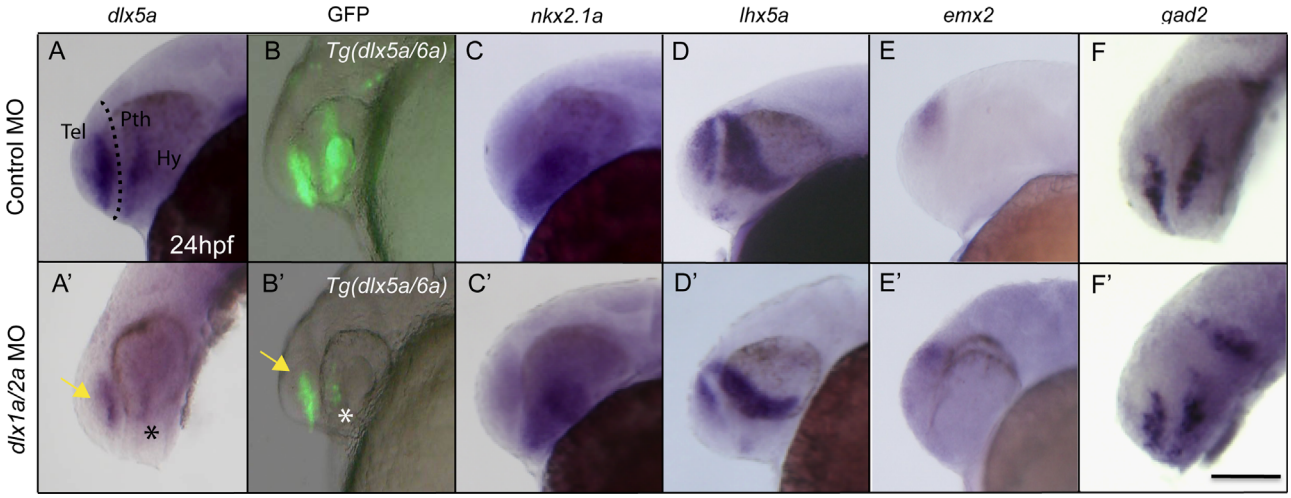

Fig. 4. Knockdown of *dlx1a/2a* identifies cross-regulatory interactions between the *dlx1a/dlx2a* and *dlx5a/dlx6a dlx* bigene clusters. (A) Double knockdown of *dlx1a/2a* results in reduced *dlx5a* expression in the telencephalon (arrow) and diencephalon (asterisk). (B) The loss of *dlx5a/6a* regulatory element activity in Tg(*dlx5a/6a*:GFP) in *dlx1a/2a* morphants is consistent with the loss of *dlx5a* expression. The defects in the prethalamus are not due to loss of mis-patterning of the structures as (C) *nkx2.1a*, (D) *emx2*, and (E) *lhx5a* genes are unaffected in *dlx1a/2a* morphants. (F) The *dlx1a/2a* morphants do not show a loss of *gad2* expression in the forebrain. Scale bar = 100 μm.

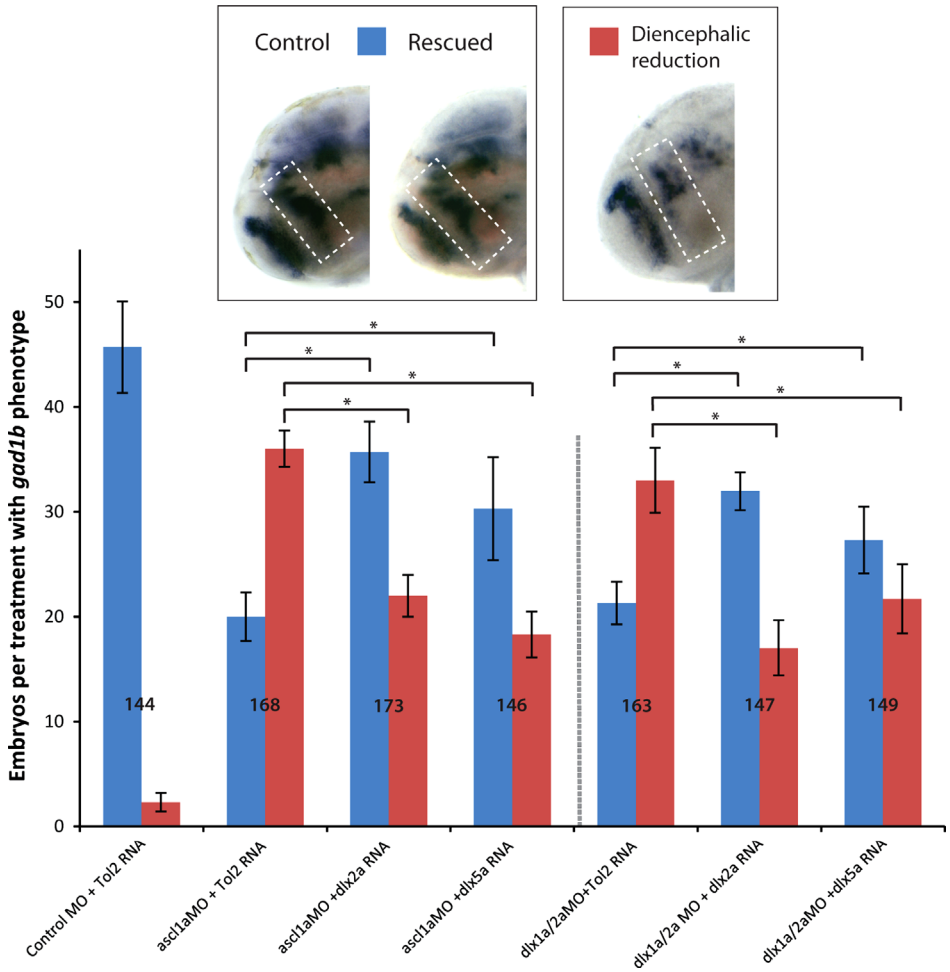

Fig. 5. Exogenous *dlx2a* and *dlx5a* mRNA expression in *ascl1a* and *dlx* morphants rescues *gad1b* expression in the diencephalon (prethalamus and hypothalamus). Average number of embryos per injected treatment with normal (blue), and reduced (red) diencephalic *gad1b* expression. A clear difference in expression is observed between the embryos injected with the control MO+Tol2 mRNA (left insert) and the embryos injected with the *ascl1a*MO+Tol2 RNA (right insert). The latter is thereafter treated as the baseline for comparison with *ascl1a*MO+*dlx2a* (middle insert). Furthermore, exogenous expression of *dlx5a*, and particularly of *dlx2a* mRNA, is able to significantly decrease the proportion of embryos with reduced *gad1b* expression in the diencephalon of *dlx1a/2a* morphants. Data from 3 experimental replicates, with total individuals per treatment shown within graph ranging from $n = 144–173$, $p < 0.05$.

prethalamus and hypothalamus (red bars in Fig. 5). A cohort of injected embryos for a given treatment would have varying proportions of these two phenotypes. Tol2 mRNA was injected as a control because this RNA is not expected to affect the ascl1a-dlx-gad1b pathway. Sixty-four percent of *ascl1a* morphants show reduced *gad1b* expression. However upon co-injection with exogenous *dlx2a* or *dlx5a* mRNA this phenotype was significantly rescued in the diencephalon of *ascl1a* morphants (Fig. 5). Similarly, 61% of *dlx1a/2a* morphants show reduced *gad1b* but exogenous expression of *dlx2a* or *dlx5a* mRNA significantly rescues this phenotype in the diencephalon (Fig. 5).

Discussion

ascl1a expression is necessary for the proper regulation of the dlx and gad1b genes

The *ascl1a* gene is co-expressed with *dlx1a*, which is later co-expressed with *dlx5a* and *gad1b* in the forebrain, reminiscent of the expression of their mouse orthologs (Porteus et al., 1994; Yun et al., 2002; Andrews et al., 2003; Stühmer et al., 2002a,b; Long et al., 2009a,b). Overlap in *ascl1a*, *dlx* and *gad1b* gene expression suggests that the GRNs necessary for GABAergic interneuron development in mammals and teleosts may be similar. In the mouse, *Ascl1* is required for the generation of early born neurons in the subcortical telencephalon, whereas *Dlx* genes play a role in late neurogenesis (Casarosa et al., 1999; Horton et al., 1999; Anderson et al., 1997a, Yun et al., 2002; Long et al., 2009a,b). The gene expression data, coupled with their relationship during neurogenesis in the forebrain suggest that *Ascl1* is upstream of *Dlx*. Knockdown of the zebrafish *Ascl1* ortholog, *ascl1a*, results in the loss of *dlx1a/2a* and *dlx5a* expression in the prethalamus and hypothalamus, and possibly lower expression in the telencephalon, showing that *ascl1a* is involved in the regulation of *dlx* genes in diencephalic territories. The loss of Ascl1a function also results in altered *gad1b* expression in the same region (Fig. 6). Although *Ascl1* mutant mice show increased *Dlx* expression due to premature differentiation of the subventricular zone (Long et al., 2009a, b), we do not see increased *dlx* expression in the telencephalon of zebrafish *ascl1a* morphants. This may also be explained by a potentially redundant function of the *gsx* gene family in the zebrafish forebrain. Whereas mouse Ascl1 is a downstream effector of Gsx function in the telencephalon (Wang et al., 2009), *gsx1* is not expressed in the zebrafish telencephalon at early developmental stages (Scholpp et al., 2007), and thus should not control *dlx* expression. It remains possible that *gsx2* may play a role in the telencephalon, however this has yet to be tested.

MO-mediated knockdown of *dlx1a/2a* leads to impaired expression of *dlx5a* and *gad1b* in the prethalamus and hypothalamus. We therefore attribute, at least in part, the loss of *dlx5a* and *gad1b* expression in the diencephalon of *ascl1a* morphants to the loss of proper *dlx1a/2a* function. Our data suggest *ascl1a* regulates *dlx1a/2a* expression, which may then regulate *dlx5a/6a* (Fig. 4) and *gad1b* expression (Fig. 6). Furthermore, the results of *dlx5a/6a* morpholino injections show a mild modification of *gad1b* expression in the ventral prethalamus when compared to *dlx1a/2a* morphants. This suggests that *dlx5a/6a* may play a relatively smaller role in the regulation of *gad1b* in this region. The rescue of the prethalamic *gad1b* phenotype in *ascl1a* morphants by exogenous expression of *dlx2a* also supports the existence of a genetic cascade consisting of *ascl1a*, *dlx1a/2a*, *dlx5a/6a*, leading to expression of the Gad1b enzyme.

Dlx function during prethalamus development in the zebrafish

The *Dlx* genes have largely redundant functions within the mouse forebrain, as suggested by the synergistic phenotypes see after combinatorial loss of *Dlx* genes (Qiu et al., 1995; Acampora et al., 1999; Anderson et al., 1997b; Marin et al., 2000; Cobos et al., 2007; Long et al., 2007; Mao et al., 2009; Long et al., 2009a,b; Wang et al., 2012). For instance, although $Dlx1^{-/-}$ and $Dlx2^{-/-}$ mutants have only subtle neural defects, $Dlx1^{-/-}/Dlx2^{-/-}$ double mutants show a major block in neurogenesis and differentiation resulting in a loss of *Gad* expression in the ventral telencephalon. Similarly, we show that knockdown of individual *dlx* genes in the zebrafish had no discernible effect on *gad1b* expression whereas double knockdowns resulted in reduced *gad1b* expression in the prethalamus and hypothalamus. Impaired *gad1b* expression is unlikely due to increased cell death or loss of the structures as specific molecular markers for this forebrain region are unaltered in *dlx* morphants.

In contrast to the prethalamus, the combined knockdown of both *dlx1a/2a* does not result in discernible loss of *gad1b* expression in the telencephalon. However, it is possible that the expression level of *gad1b* is altered but is not detectable with our

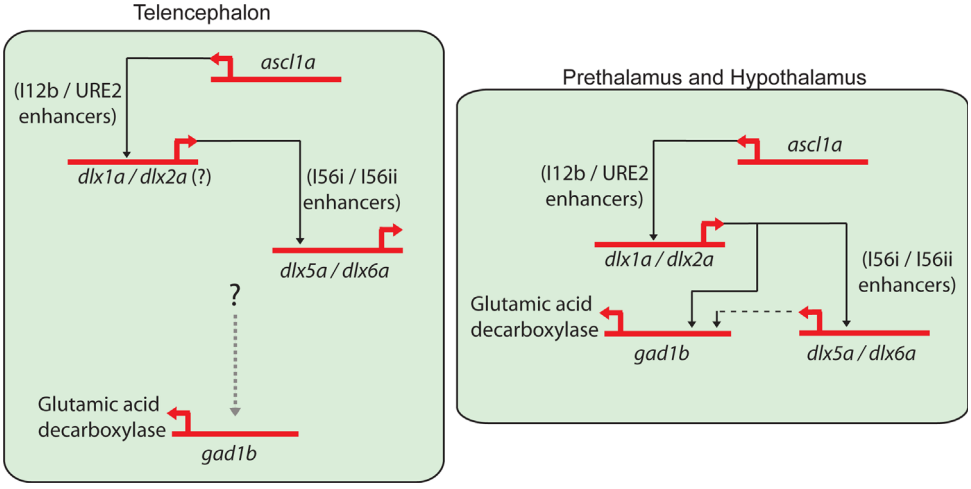

Fig. 6. Model showing the regulation of the *dlx* and *gad1b* genes in the zebrafish forebrain. In the telencephalon, *ascl1a* controls *Tg(dlx1URE2:GFP)* activity and may modulate *dlx1a/2a* expression. The *dlx1a/2a* genes will regulate *dlx5a* expression via its CREs. A currently unknown genetic pathway controls the expression of *gad1b* in the telencephalon as knockdown of *ascl1a*, *dlx1a/2a* or *dlx5a/6a* has little or no effect on transcription. In the diencephalon, specifically the ventral part of the prethalamus and the hypothalamus, *ascl1a* controls the expression of *dlx1a/2a* which then regulates *dlx5a/6a* and *gad1b* in this tissue. The *dlx5a/6a* genes may play a minor role in *gad1b* regulation in the diencephalon (as shown by dashed black line).

methods. The presence of *dlx2b*, a teleost specific paralog of *dlx2a* that is co-expressed with *dlx1a* and *dlx2a* in the forebrain, could explain the absence of *gad1b* phenotype in the telencephalon. However, MO-mediated knock down of *dlx2b* did not affect forebrain *gad1b* expression and the combined administration of MOs for *dlx1a*, *dlx2a* and *dlx2b* did not enhance the phenotype observed with the *dlx1a*/*2a* MO combination. The lack of a *gad1b* phenotype in the zebrafish telencephalon points to differences in the genetic pathways regulating *gad1b* expression in this tissue between teleosts and mammals.

Dlx1$^{-/-}$/*Dlx2*$^{-/-}$ mutant mice show a loss of *Gad1* expression in the olfactory bulb and the ventral telencephalon (Anderson et al., 1997a,b; Bulfone et al., 1998, Long et al., 2007; Long et al., 2009a,b; Wang et al., 2012). Furthermore, ectopic expression of the *Dlx* genes leads to expression of *Gad* genes, *Gad1* and *Gad2*, in mouse telencephalon slice cultures (Anderson et al., 1999; Stühmer et al., 2002a).The knockdown of *dlx5a*/*6a* causes less dramatic *gad1b* changes in the ventral prethalamus. This suggests that *dlx1a*/*2a* function may be sufficient for *gad1b* expression in this region. Unfortunately, the mouse *Dlx5*$^{-/-}$/*Dlx6*$^{-/-}$ mutant forebrain phenotype cannot be fully studied due to exencephaly (Robledo et al., 2002, Wang et al., 2010). However, our morpholino data may provide some clarity as to the role of Dlx1/2 and/or Dlx5/6 for the GABAergic neuron differentiation and migration in the forebrain. Due to the close genetic interactions between *Dlx* genes, it has been challenging to uncouple their functions. Our data suggests that Dlx1/2 function is critical for *gad1b* expression, whereas Dlx5/6 may have a relatively minor role.

Loss of *dlx* function does not affect the expression of *gad2*. Distinct genetic pathways may regulate the *gad1b* and *gad2* genes (as suggested by Szabó et al. (1996), Pinal et al. (1997) and Yanagawa et al. (1997)) although some upstream factors such as *fgf3*/*8* and *her6* were shown to be necessary for the proper expression of both *gad1b* and *gad2* in the prethalamus (Miyake et al., 2005; Scholpp et al., 2009).

Regulatory interactions involving Dlx genes

In mammals, the Dlx proteins participate in auto- and cross-regulatory interactions by binding regulatory elements both within their own bigene cluster and in paralogous *Dlx* cluster (Zerucha et al., 2000; Zhou et al., 2004; Bond et al., 2009; Potter et al., 2009). Altered Dlx binding to such regulatory elements results in the loss of reporter gene expression in the forebrain of transgenic mice (Zerucha et al., 2000; Poitras et al., 2007). To explore this issue in the zebrafish, we tested the effect of the *ascl1a* and *dlx* MO injections on transgenic lines where the reporter gene (*gfp*) is under the control of zebrafish CREs from the *dlx* loci. The *Tg*(*dlx1a*/*2aIG*:*GFP*) contains the I12b element and is active in telencephalon and prethalamus, similarly to its orthologous mouse sequences (Ghanem et al., 2003; Ghanem et al., 2007; Poitras et al., 2007; MacDonald et al., 2010a). Furthermore this regulatory element contains a highly conserved ASCL1 binding site (E box) that that is essential for enhancer activity (Poitras et al., 2007). Expression of *Tg*(*dlx1a*/*2aIG*:*GFP*) is impaired in the diencephalon of *ascl1a* morphants with no severe impact in the telencephalon. The zebrafish URE2 CRE found upstream of *dlx1a* does not contain a conserved E-box (MacDonald, R.B. and Ekker, M., Unpublished observations) and may not require Ascl1a for its activity. Surprisingly, expression of the URE2-containing *Tg*(*dlx1aURE2*:*GFP*) transgene is impaired in both the telencephalon and diencephalon of *ascl1a* morphants. This large effect contrasts with changes in *dlx1a*/*2a* mRNA expression that were observed only in the diencephalon of morphants. Similarly, *Tg*(*dlx5a*/*6a*:*GFP*) expression was severely affected in *ascl1a* morphants without a comparable loss of *dlx5a* or *dlx6a* mRNA expression. These apparent discrepancies are likely attributable to the redundancy in gene regulatory controls and/or would suggest influence of factors, in addition to Ascl1a, involved in *dlx* regulation.

Lineage-specific changes GRNs in the forebrain of mice and zebrafish

The early patterning of the diencephalon seems to have been highly conserved amongst extant vertebrates. The roles of Shh, Fez, Otx, Wnts, Ascl1, Neurog1 and FGFs in prethalamic and thalamic regionalization are similar in the mouse, chick, *Xenopus* and zebrafish (Scholpp and Lumsden, 2010). Our results suggest that regulatory relationships between *ascl1a*, *dlx1a*/*2a*, *dlx5a*/*6a* and *gad1b* are present in the developing zebrafish prethalamus and hypothalamus, but the position of *dlx5a* and *dlx6a* in such a pathway remains ambiguous due to a smaller dlx5a/6a knock down effect on gad1b expression. The loss *Dlx5* and *Dlx6* expression in the forebrain of *Dlx1*$^{-/-}$/*Dlx2*$^{-/-}$ mice and the regulatory relationships between them (Zerucha et al., 2000; Anderson et al., 1997a) support an implication of *Dlx5*/*Dlx6* in GABAergic neuron development. It is possible that the *Dlx5*/*Dlx6* genes of the last common ancestor of mice and zebrafish were involved in such a developmental pathway, but this role became less important in the lineage leading to zebrafish. If this were the case, *dlx5a*/*6a* may have been examples of nodes in a GRN that were more vulnerable to exclusion, perhaps due to regulatory redundancy with other *dlx* genes.

The current study shows the conservation of the diencephalic GRN regulating GABAergic interneuron development and the apparent divergence of the same process in the telencephalon. Our observations reinforce the neuromeric model of brain development and evolution (Rubenstein et al., 1994; Hauptmann and Gerster, 2000; Puelles and Rubenstein, 2003). This model postulates that the early vertebrate forebrain is composed of relatively discrete morphogenetic units termed neuromeres, between which cellular migration is limited, and within each neuromere developmental GRNs may undergo dynamic evolutionary changes. It is possible that during the approximately 300 million since the divergence of lineages leading to mice and zebrafish, the GRN underlying GABAergic interneuron specification in the telencephalic neuromere has changed. Our work suggests that divergence in the activity of GRNs responsible for forebrain neurodevelopment has occurred between mice and zebrafish vertebrate lineages.

Acknowledgments

The authors would like to thank Dr. Stephen Wilson for his generous gifts of the *ascl1a*$^{5'UTR}$ MO and *ascl1a* plasmid. The authors would also like to thank Dr. Charles Kimmel for *dlx5a* splice blocking MOs and fluorescent *in situ* reagents. This work was supported by CIHR Grant MOP14460 to ME and NIH Grants RO1DE13834 and PO1HD22486 to Charles Kimmel, University of Oregon.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.05.025.

References

Acampora, D., Merlo, G.R., Paleari, L., Zerega, B., Postiglione, M.P., Mantero, S., Bober, E., Barbieri, O., Simeone, A., Levi, G., 1999. Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene Dlx5. Development 126 (17), 3795–3809.

Akimenko, M.A., Ekker, M., Wegner, J., Lin, W., Westerfield, M., 1994. Combinatorial expression of three zebrafish genes related to *distal-less*: part of a homeobox gene code for the head. J. Neurosci. 14, 3475–3486.
Allende, M.L., Weinberg, E.S., 1994. The expression pattern of two zebrafish achaete-scute homolog (ash) genes is altered in the embryonic brain of the cyclops mutant. Dev. Biol. 166, 509–530.
Allan, D.W., Thor, S., 2003. Together at last: BHLH and LIM-HD Regulators cooperate to specify motor neurons. Neuron 38, 675–677.
Anderson, S.A., Qiu, M., Bulfone, A., Eisenstat, D.D., Meneses, J., Pedersen, R., Rubenstein, J.L.R., 1997a. Mutations of the homeobox genes *Dlx-1* and *Dlx-2* disrupt the striatal subventricular zone and differentiation of late-born striatal neurons. Neuron 19, 27–37.
Anderson, S.A., Eisenstat, D.D., Shi, L., Rubenstein, J.L.R., 1997b. Interneuron migration from basal forebrain to neocortex: dependence on *Dlx* genes. Science 278, 474–476.
Anderson, S., Mione, M., Yun, K., Rubenstein, J.L., 1999. Differential origins of neocortical projection and local circuit neurons: role of Dlx genes in neocortical interneuronogenesis. Cereb. Cortex 9 (6), 646–654.
Andrews, G.L., Yun, K., Rubenstein, J.L., Mastick, G.S., 2003. Dlx transcription factors regulate differentiation of dopaminergic neurons of the ventral thalamus. Mol. Cell. Neurosci. 23, 107–120.
Bertrand, N., Castro, D.S., Guillemot, F., 2002. Proneural genes and the specification of neural cell types. Nat. Rev. Neurosci. 3, 517–530.
Bond, A.M., Vangompel, M.J., Sametsky, E.A., Clark, M.F., Savage, J.C., Disterhoft, J.F., Kohtz, J.D., 2009. Balanced gene regulation by an embryonic brain ncRNA is critical for adult hippocampal GABA circuitry. Nat. Neurosci. 12, 1020–1027.
Bulfone, A., Wang, F., Hevner, R., Anderson, S., Cutforth, T., Chen, S., Meneses, J., Pedersen, R., Axel, R., Rubenstein, J.L., 1998. An olfactory sensory map develops in the absence of normal projection neurons or GABAergic interneurons. Neuron 21, 1273–1282.
Casarosa, S., Fode, C., Guillemot, F., 1999. Mash1 regulates neurogenesis in the ventral telencephalon. Development 126, 525–534.
Cau, E., Gradwohl, G., Casarosa, S., Kageyama, R., Guillemot, F., 2000. Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium. Development 127, 2323–2332.
Cau, E., Wilson, S.W., 2003. Ash1a and neurogenin1 function downstream of floating head to regulate epiphysial neurogenesis. Development 130, 2455–2466.
Cobos, I., Borello, U., Rubenstein, J.L.R., 2007. Dlx Transcription factors promote migration through repression of axon and dendrite growth. Neuron 54, 873–888.
Davidson, E.H., Levine, M.S., 2008. Properties of developmental gene regulatory networks. Proc. Natl. Acad. Sci. USA 105, 20063–20066.
Depew, M.J., Lufkin, T., Rubenstein, J.L., 2002. Specification of jaw subdivisions by Dlx genes. Science 298 (5592), 381–385.
Dorsky, R.I., Sheldahl, L.C., Moon, R.T., 2002. A transgenic Lef1/beta-catenin dependent reporter is expressed in spatially restricted domains throughout zebrafish development. Dev. Biol. 241 (2), 229–237.
Eisenstat, D.D., Liu, J., Mione, M., Zhong, W., Yu, G., Anderson, S.A., Ghattas, I., Puelles, L., Rubenstein, J.L.R., 1999. DLX-1, DLX-2 and DLX-5 expression define distinct stages of basal forebrain differentiation. J. Comp. Neurol. 414, 217–237.
Ellies, D.L., Stock, D.W., Hatch, G., Giroux, G., Weiss, K.M., Ekker, M., 1997. Relationship between the genomic organization and the overlapping embryonic expression patterns of the zebrafish *Dlx* genes. Genomics 45, 580–590.
Fausett, B.V., Gumerson, J.D., Goldman, D., 2008. The proneural basic helix–loop–helix gene ascl1a is required for retina regeneration. J. Neurosci. 28, 1109–1117.
Fode, C., Ma, Q., Casarosa, S., Ang, S.L., Anderson, D.J., Guillemot, F., 2000. A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. Genes Dev. 14, 67–80.
Ghanem, N., Jarinova, O., Amores, A., Hatch, G., Park, B.K., Rubenstein, J.L.R., Ekker, M., 2003. Regulatory roles of conserved intergenic domains in vertebrate *Dlx* bigene clusters. Genome Res. 13, 533–543.
Ghanem, N., Yu, M., Long, J., Hatch, G., Rubenstein, J.L., Ekker, M., 2007. Distinct cis-regulatory elements from the Dlx1/Dlx2 locus mark different progenitor cell populations in the ganglionic eminences and different subtypes of adult cortical interneurons. J. Neurosci. 27, 5012–5022.
Guillemot, F., Joyner, A.L., 1993. Dynamic expression of the murine achaete-scute homologue Mash-1 in the developing nervous system. Mech. Dev. 42, 171–185.
Hauptmann, G., Gerster, T., 2000. Regulatory gene expression patterns reveal transverse and longitudinal subdivisions of the embryonic zebrafish forebrain. Mech. Dev. 91 (1–2), 105–118.
Herzog, W., Sonntag, C., Walderich, B., Odenthal, J., Maischein, H.M., Hammerschmidt, M., 2004. Genetic analysis of adenohypophysis formation in zebrafish. Mol. Endocrinol. 18, 1185–1195.
Horton, S., Meredith, A., Richardson, J.A., Johnson, J.E., 1999. Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. Mol. Cell. Neurosci. 14, 355–369.
Jackman, W.R., Stock, D.W., 2006. Transgenic analysis of Dlx regulation in fish tooth development reveals evolutionary retention of enhancer function despite organ loss. Proc. Natl. Acad. Sci. USA 103, 19390–19395.
Jeong, J., Li, X., McEvilly, R.J., Rosenfeld, M.G., Lufkin, T., Rubenstein, J.L., 2008. Dlx genes pattern mammalian jaw primordium by regulating both lower jaw-specific and upper jaw-specific genetic programs. Development 135 (17), 2905–2916.
Jowett, T., Yan, Y.L., 1996. Double fluorescent *in situ* hybridization to zebrafish embryos. Trends Genet. 12, 387–389.
Kadonaga, J.T., 2004. Regulation of RNA Polymerase II transcription by sequence-specific DNA binding factors. Cell 116, 247–257.
Kaji, T., Artinger, K.B., 2004. Dlx3b and dlx4b function in the development of Rohon–Beard sensory neurons and trigeminal placode in the zebrafish neurula. Dev. Biol. 276, 523–540.
Kulkarni, M.M., Arnosti, D.N., 2003. Information display by transcriptional enhancers. Development 130, 6569–6575.
Lauter, G, Söll, I., Hauptmann, G., 2013. Molecular characterization of prosomeric and intraprosomeric subdivisions of the embryonic zebrafish diencephalon. J. Comp. Neurol. 521 (5), 1093–1118.
Levine, M., Davidson, E.H., 2005. Gene regulatory networks for development. Proc. Natl. Acad. Sci. USA 102, 4936–4942.
Liu, J.K., Ghattas, I., Liu, S., Chen, S., Rubenstein, J.L.R., 1997. Dlx genes encode DNA-binding proteins that are expressed in an overlapping and sequential pattern during basal ganglia differentiation. Dev. Dyn. 210, 498–512.
Lo, L.C., Johnson, J.E., Wuenschell, C.W., Saito, T., Anderson, D.J., 1991. Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. Genes Dev. 5, 1524–1537.
Long, J.E., Garel, S., Alvarez-Dolado, M., Yoshikawa, K., Osumi, N., Alvarez-Buylla, A., Rubenstein, J.L., 2007. Dlx-dependent and -independent regulation of olfactory bulb interneuron differentiation. J. Neurosci. 27 (12), 3230–3243.
Long, J.E., Swan, C., Liang, W.S., Cobos, I., Potter, G.B., Rubenstein, J.L., 2009a. Dlx1&2 and Mash1 transcription factors control striatal patterning and differentiation through parallel and overlapping pathways. J. Comp. Neurol. 512 (4), 556–572.
Long, J.E., Cobos, I., Potter, G.B., Rubenstein, J.L., 2009b. Dlx1&2 and Mash1 transcription factors control MGE and CGE patterning and differentiation through parallel and overlapping pathways. Cereb. Cortex 19, i96–i106.
MacDonald, R.B., Debiais-Thibaud, M., Talbot, J.C., Ekker, M., 2010a. The relationship between *dlx* and *gad1* expression indicates highly conserved genetic pathways in the zebrafish forebrain. Dev. Dyn. 239 (8), 2298–2306.
MacDonald, R.B., Debiais-Thibaud, M., Martin, K., Tay, B.H., Venkatesh, B., Ekker, M., 2010b. Functional conservation of a forebrain enhancer from the elephant shark (*Callorhinchus milii*) in zebrafish and mice. BMC Evol. Biol. 10, 157.
Mao, R., Page, D.T., Merzlyak, I., Kim, C., Tecott, L.H., Janak, P.H., Rubenstein, J.L., Sur, M., 2009. Reduced conditioned fear response in mice that lack Dlx1 and show subtype-specific loss of interneurons. J. Neurodev. Disord. 1 (3), 224–236.
Marin, O., Anderson, S.A., Rubenstein, J.L., 2000. Origin and molecular specification of striatal interneurons. J. Neurosci. 20, 6063–6076.
Martin, S.C., Heinrich, G., Sandell, J.H., 1998. Sequence and expression of glutamic acid decarboxylase isoforms in the developing zebrafish. J. Comp. Neurol. 396, 253–266.
Mione, M., Baldessari, D., Deflorian, G., Nappo, G., Santoriello, C., 2008. How neuronal migration contributes to the morphogenesis of the CNS: insights from the zebrafish. Dev. Neurosci. 30, 65–81.
Miyake, A., Nakayama, Y., Konishi, M., Itoh, N., 2005. Fgf19 regulated by Hh signaling is required for zebrafish forebrain development. Dev. Biol. 288 (1), 259–275.
Morita, T., Nitta, H., Kiyama, Y., Mori, H., Mishina, M., 1995. Differential expression of two zebrafish Emx homeoprotein mRNAs in the developing brain. Neurosci. Lett. 198, 131–134.
Mueller, T., Wullimann, M.F., Guo, S., 2008. Early teleostean basal ganglia development visualized by zebrafish Dlx2a, Lhx6, Lhx7, Tbr2 (Eomesa), and GAD67 gene expression. J. Comp. Neurol. 507, 1245–1257.
Panne, D., 2008. The Enhanceosome. Curr. Opin. Struct. Biol. 18, 236–242.
Pinal, C.S., Cortessis, V., Tobin, A.J., 1997. Multiple elements regulate GAD65 transcription. Dev. Neurosci. 19 (6), 465–475.
Peukert, D., Weber, S., Lumsden, A., Scholpp, S., 2011. Lhx2 and Lhx9 determine neuronal differentiation and compartition in the caudal forebrain by regulating Wnt signaling. PLoS Biol. 9 (12), e1001218.
Pogoda, H.M., von der Hardt, S., Herzog, W., Kramer, C., Schwarz, H., Hammerschmidt, M., 2006. The proneural gene ascl1a is required for endocrine differentiation and cell survival in the zebrafish adenohypophysis. Development 133, 1079–1089.
Poitras, L., Ghanem, N., Hatch, G., Ekker, M., 2007. The proneural determinant MASH1 regulates forebrain Dlx1/2 expression through the I12b intergenic enhancer. Development 134, 1755–1765.
Porteus, M.H., Bulfone, A., Liu, J.K., Puelles, L., Lo, L.C., Rubenstein, J.L., 1994. DLX-2, MASH-1, and MAP-2 expression and bromodeoxyuridine incorporation define molecularly distinct cell populations in the embryonic mouse forebrain. J. Neurosci. 14, 6370–6383.
Potter, G.B., Petryniak, M.A., Shevchenko, E., McKinsey, G.L., Ekker, M., Rubenstein, J. L., 2009. Generation of Cre-transgenic mice using Dlx1/Dlx2 enhancers and their characterization in GABAergic interneurons. Mol. Cell. Neurosci. 40, 167–186.
Puelles, L., Rubenstein, J.L., 2003. Forebrain gene expression domains and the evolving prosomeric model. Trends Neurosci. 26 (9), 469–476.
Qiu, M., Bulfone, A., Martinez, S., Meneses, J.J., Shimamura, K., Pedersen, R.A., Rubenstein, J.L., 1995. Null mutation of Dlx-2 results in abnormal morphogenesis of proximal first and second branchial arch derivatives and abnormal differentiation in the forebrain. Genes Dev. 9 (20), 2523–2538.
Robledo, R.F., Rajan, L., Li, X., Lufkin, T., 2002. The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. Genes Dev. 16 (9), 1089–1101.
Rohr, K.B., Concha, M.L., 2000. Expression of nk2.1a during early development of the thyroid gland in zebrafish. Mech. Dev. 95, 267–270.
Rubenstein, J.L., Martinez, S., Shimamura, K., Puelles, L., 1994. The embryonic vertebrate forebrain: the prosomeric model. Science 266 (5185), 578–580.

Scholpp, S., Foucher, I., Staudt, N., Peukert, D., Lumsden, A., Houart, C., 2007. Otx1l, Otx2 and Irx1b establish and position the ZLI in the diencephalon. Development 134 (17), 3167–3176.
Scholpp, S., Delogu, A., Gilthorpe, J., Peukert, D., Schindler, S., Lumsden, A., 2009. Her6 regulates the neurogenetic gradient and neuronal identity in the thalamus. Proc. Natl. Acad. Sci. USA 106 (47), 19895–19900.
Scholpp, S., Lumsden, A., 2010. Building a bridal chamber: development of the thalamus. Trends Neurosci. 33 (8), 373–380.
Solomon, K.S., Fritz, A., 2002. Concerted Action of two *Dlx* paralogs in sensory placode formation. Development 129, 3127–3136.
Sperber, S.M., Saxena, V., Hatch, G., Ekker, M., 2008. Zebrafish dlx2a contributes to hindbrain neural crest survival, is necessary for differentiation of sensory ganglia and functions with dlx1a in maturation of the arch cartilage elements. Dev. Biol. 314, 59–70.
Stühmer, T., Anderson, S.A., Ekker, M., Rubenstein, J.L.R., 2002a. Ectopic expression of the *Dlx* genes induces glutamic acid decarboxylase and *Dlx* expression. Development 129, 245–252.
Stühmer, T., Puelles, L., Ekker, M., Rubenstein, J.L., 2002b. Expression from a Dlx gene enhancer marks adult mouse cortical GABAergic neurons. Cereb. Cortex 12, 75–85.
Szabó, G., Katarova, Z., Körtvély, E., Greenspan, R.J., Urbán, Z., 1996. Structure and the promoter region of the mouse gene encoding the 67-kD form of glutamic acid decarboxylase. DNA Cell Biol. 15 (12), 1081–1091.
Talbot, J.C., Johnson, S.L., Kimmel, C.B., 2010. hand2 and Dlx genes specify dorsal, intermediate and ventral domains within zebrafish pharyngeal arches. Development 137 (15), 2507–2517.
Thisse, C., Thisse, B., 1998. High resolution whole-mount *in situ* hybridization. Zebrafish Science Monitor 5, 8–9.
Toyama, R., Curtiss, P.E., Otani, H., Kimura, M., Dawid, I.B., Taira, M., 1995. The LIM class homeobox gene lim5: implied role in CNS patterning in xenopus and zebrafish. Dev. Biol. 170, 583–593.
Walker, M.B., Miller, C.T., Talbot, J.C., Stock, D.W., Kimmel, C.B., 2006. Zebrafish *furin* mutants reveal intricacies in regulating Endothelin1 signaling in craniofacial patterning. Dev. Biol. 295, 194–205.
Wang, B., Waclaw, R.R., Allen, Z.J., Guillemot, F., Campbell, K., 2009. Ascl1 is a required downstream effector of Gsx gene function in the embryonic mouse telencephalon. Neural. Dev. 4 (5), http://dx.doi.org/10.1186/1749–8104-4–5.
Wang, Y., Dye, C.A., Sohal, V., Long, J.E., Estrada, R.C., Roztocil, T., Lufkin, T., Deisseroth, K., Baraban, S.C., Rubenstein, J.L.R., 2010. *Dlx5* and *Dlx6* regulate the development of parvalbumin-expressing cortical interneurons. J. Neurosci. 30, 5334–5345.
Wang, B., Long, J.E., Flandin, P., Pla, R., Waclaw, R.R., Campbell, K., Rubenstein, J.L., 2012. Loss of Gsx1 and Gsx2 function rescues distinct phenotypes in Dlx1/2 mutants. J. Comp. Neurol. 521 (7), 1561–1584.
Welten, M.C., de Haan, S.B., van den Boogert, N., Noordermeer, J.N., Lamers, G.E., Spaink, H.P., Meijer, A.H., Verbeek, F.J., 2006. ZebraFISH: fluorescent *in situ* hybridization protocol and three-dimensional imaging of gene expression patterns. Zebrafish 3, 465–476.
Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory use of Zebrafish (Danio Rerio). University of Oregon Press, Eugene, Oregon.
Wullimann, M.F., Mueller, T., 2002. Expression of Zash-1a in the postembryonic zebrafish brain allows comparison to mouse Mash1 domains. Brain Res. Gene Expr. Patterns 1, 187–192.
Yanagawa, Y., Kobayashi, T., Kamei, T., Ishii, K., Nishijima, M., Takaku, A., Tamura, S., 1997. Structure and alternative promoters of the mouse glutamic acid decarboxylase 67 gene. Biochem. J. 326 (Pt2), 573–578.
Yang, L., Zhang, H., Hu, G., Wang, H., Abate-Shen, C., Shen, M.M., 1998. An early phase of embryonic Dlx5 expression defines the rostral boundary of the neural plate. J. Neurosci. 18, 8322–8330.
Yun, K., Fischman, S., Johnson, J., Hrabe de Angelis, M., Weinmaster, G., Rubenstein, J.L., 2002. Modulation of the notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon. Development 129, 5029–5040.
Zerucha, T., Stühmer, T., Hatch, G., Park, B.K., Long, Q., Yu, G., Gambarotta, A., Schultz, J.R., Rubenstein, J.L.R., Ekker, M., 2000. A highly conserved enhancer in the *Dlx5/Dlx6* intergenic region is the site of cross-regulatory interactions between *DLx* genes in the embryonic forebrain. J. Neurosci. 20, 709–721.
Zhou, Q.P., Le, T.N., Qiu, X., Spencer, V., de Melo, J., Du, G., Plews, M., Fonseca, M., Sun, J.M., Davie, J.R., Eisenstat, C, 2004. Identification of a direct Dlx homeodomain target in the developing mouse forebrain and retina by optimization of chromatin immunoprecipitation. Nucleic Acids Res. 32, 884–892.